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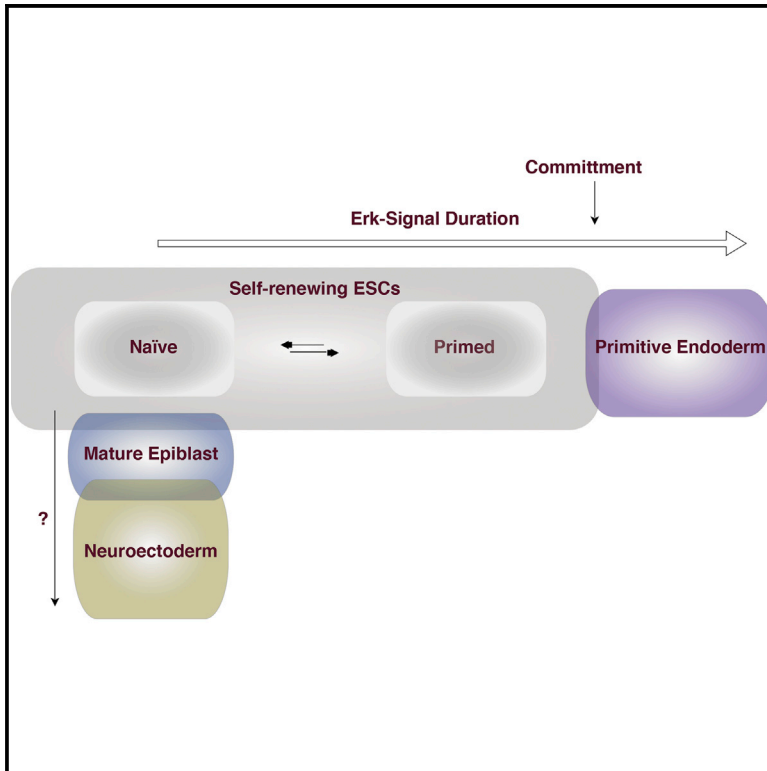
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# Cell Reports

## Erk Signaling Suppresses Embryonic Stem Cell Self-Renewal to Specify Endoderm

### Graphical Abstract



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### In Brief

Hamilton and Brickman show that Erk activity is not required for epiblast nor neural differentiation but promotes primitive endoderm priming and differentiation through suppression of a subset of the ESC gene regulatory network. The duration of Erk signaling determines the difference between reversible priming and differentiation.

### Highlights

- Erk activity is dispensable for exit from pluripotency and neural differentiation
- Erk activity suppresses pluripotency gene expression to induce endoderm specification
- Erk signaling duration determines the difference between priming and differentiation
- Nanog blocks Gata6 induction but not the inhibition of pluripotency by Erk



# Erk Signaling Suppresses Embryonic Stem Cell Self-Renewal to Specify Endoderm

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## SUMMARY

Fgf signaling via Erk activation has been associated with both neural induction and the generation of a primed state for the differentiation of embryonic stem cells (ESCs) to all somatic lineages. To dissect the role of Erk in both ESC self-renewal and lineage specification, we explored the requirements for this pathway in various in vitro differentiation settings. A combination of pharmacological inhibition of Erk signaling and genetic loss of function reveal a role for Erk signaling in endodermal, but not neural differentiation. Neural differentiation occurs normally despite a complete block to Erk phosphorylation. In support of this, Erk activation in ESCs derepresses primitive endoderm (PrE) gene expression as a consequence of inhibiting the pluripotent/epiblast network. The early response to Erk activation correlates with functional PrE priming, whereas sustained Erk activity results in PrE differentiation. Taken together, our results suggest that Erk signaling suppresses pluripotent gene expression to enable endodermal differentiation.

## INTRODUCTION

Mouse embryonic stem cells (ESCs) are immortal, karyotypically stable cell lines derived from the inner cell mass (ICM) or early epiblast of preimplantation embryos (Evans and Kaufman, 1981; Martin, 1981; Najm et al., 2011). They are capable of maintaining their differentiation potential through multiple rounds of division, of differentiating into all the lineages of the future conceptus when reintroduced into a developing embryo (Morgani et al., 2013; Robertson et al., 1986), and of undergoing directed differentiation in vitro. ESCs are defined by these functional properties, self-renewal, and pluri- (and in some cases toti-) potency, but they are also characterized by the expression of an array of genes, primarily transcription factors such as Nanog, Oct4, Rex1 (Chambers et al., 2003; Mitsui et al., 2003; Nichols et al., 1998; Takahashi and Yamanaka, 2006), and cell-surface markers including SSEA1 and PECAM1 (Canham et al., 2010; Rugg-Gunn et al., 2012).

The maintenance of ESCs in an undifferentiated state is dependent on external signals such as leukemia inhibitory factor

(LIF) (Smith et al., 1988) and BMP4 (Ying et al., 2003a). It is thought that these signals converge on a core network of transcription factors that cooperate to maintain an undifferentiated state (Martello et al., 2012, 2013). Recently, it has been shown that ESCs can be maintained in a minimal defined culture system through the combinatorial inhibition of both Mek-Erk and Gsk3 signaling (Ying et al., 2008). This condition, known as 2i, was shown to be highly effective at supporting pluripotent ESCs and, when supplemented with LIF, contains a subpopulation of single cells that exhibit totipotency (Morgani et al., 2013). The rationale behind this culture system is that robust Erk activity (downstream of Fgf4) is essential for multilineage differentiation of ESCs (Kunath et al., 2007; Ying et al., 2008), and therefore inhibiting it promotes self-renewal. The suppression of Gsk3 activity was believed to enhance the viability of undifferentiated cells grown in these defined conditions (Ying et al., 2008). However, recent studies suggest that Gsk3 activity regulates a crucial axis of the pluripotency network and can regulate self-renewal independently of Erk inhibition or LIF-Stat3 activation (Wray et al., 2011).

Although it has been proposed that Erk activity is required for differentiation toward derivatives of all three germ layers in vitro (Kunath et al., 2007; Ying et al., 2008), in vivo studies into various mutants of the Fgf-Erk pathway indicate this pathway is required for early extraembryonic endoderm differentiation (Chazaud et al., 2006; Kang et al., 2013), in addition to playing a role in aiding the survival of differentiated epiblast derived tissues (Arman et al., 1998; Feldman et al., 1995; Wilder et al., 1997). It has also been suggested that Erk activity is required for differentiation toward neural tissues in vitro (Kunath et al., 2007; Stavridis et al., 2007), although the in vivo evidence for a requirement for Fgf-Erk activity in the early stages of neural specification is controversial (Di-Gregorio et al., 2007). Experiments in chick and *Xenopus* disagree as to whether naive ectoderm can undergo neural induction merely as a result of BMP antagonism, the default model, or whether neural induction is mediated via an Fgf-Erk signal (Linker and Stern, 2004). Data from ESCs were seen to support such a role for Fgf-Erk in neural induction, although it was recently shown that Erk2, the primary Erk isozyme expressed in ESCs, is entirely dispensable for their multilineage differentiation (Hamilton et al., 2013). Moreover, although the inhibition of Erk activity enhances the differentiation of certain neural lineages when differentiated from mouse epiblast stem cells (EpiSCs) (Jaeger et al., 2011), Erk activity is still thought to be required for the progression of undifferentiated cells into a primed epiblast state, the first stage in neural differentiation.

Because self-renewal and differentiation involve the regulation of the same developmental step, just in opposing directions, we sought to examine the role of Erk in this process. We found that Erk activity itself is dispensable for neural induction and epiblast differentiation from ESCs but is absolutely required for PrE differentiation. We also found that reduced Erk activity facilitated the enhanced expression of a range of pluripotency-associated transcripts under conditions that would normally support PrE differentiation. To probe the role of Erk in promoting PrE differentiation, we designed an inducible system for Erk activation. We found that our previously described functional PrE priming was a rapid and primary response to Erk activation, and we deciphered the transcriptional hierarchy involved in this process. Thus, in ESCs, as in the blastocyst, Erk signaling regulates PrE lineage resolution and demonstrates that the primary response to this pathway is the downregulation of the pluripotency network as cells embark on a program of endoderm differentiation.

## RESULTS

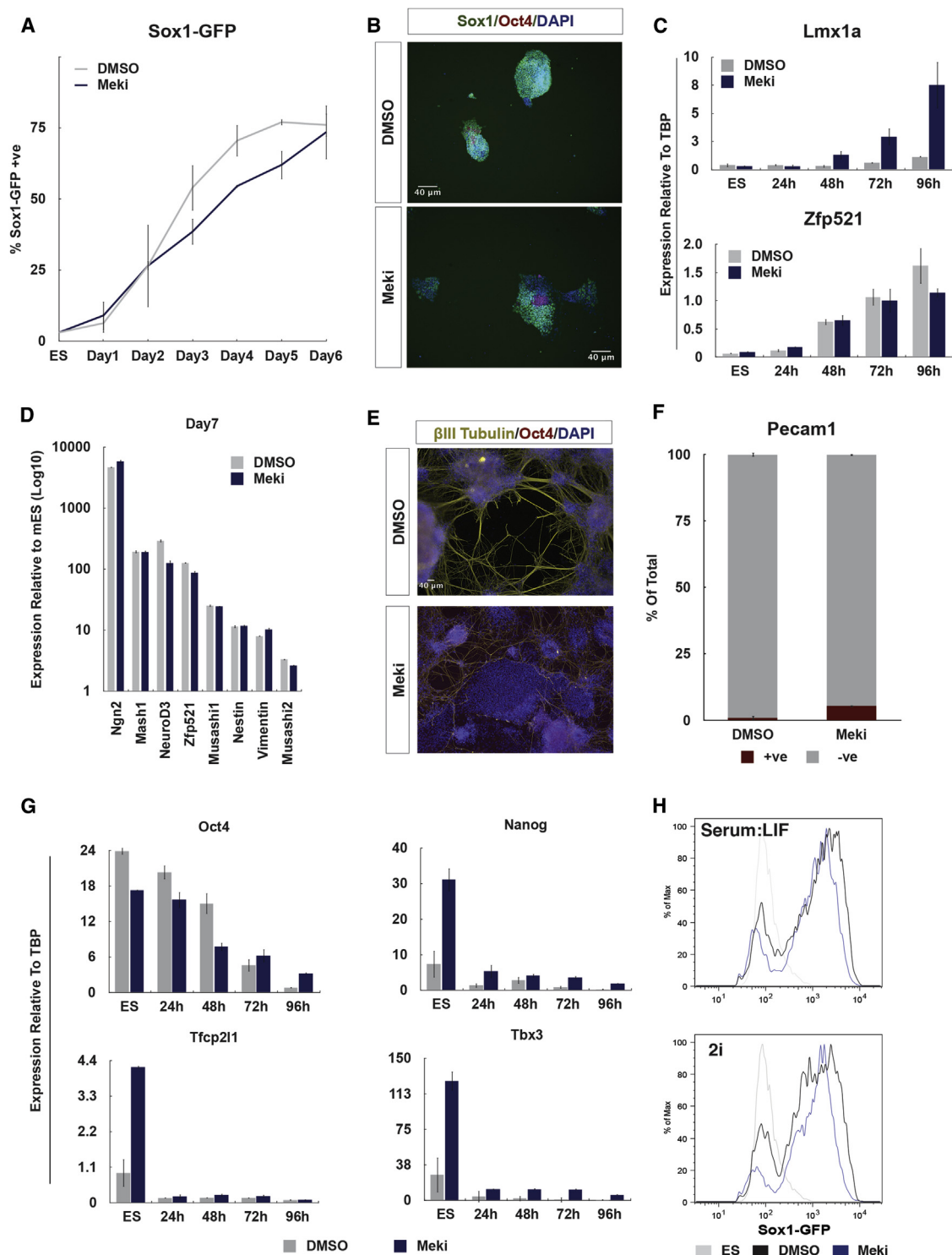
### Erk Activity Is Dispensable for Neural Induction from ESCs

Although it is broadly assumed that Erk activity is required for all epiblast differentiation, the only genetic evidence for a role for Erk signaling in ESC differentiation toward neural tissue comes from experiments using Erk2 knockout ESCs, derived from C56B6/129Ola f1 crosses (Kunath et al., 2007), though it was subsequently shown that these cells could differentiate in embryoid bodies (Hamilton et al., 2013). When Erk2 is knocked out on a pure 129 background, homozygous mutant ESC can undergo normal neural differentiation (Hamilton et al., 2013). However, these cells are not Erk deficient because they have compensatory Erk1 activity. Moreover, because spontaneous neural differentiation has been observed in ESC cultures maintained in minimal media (N2B27) containing only a small molecule block to Erk/Mek signaling (Wray et al., 2011; Figure 4C), we sought to reanalyze the requirement for Erk activity during the early stages of neural differentiation. We took advantage of the well-characterized Sox1-GFP reporter line, 46C (Ying et al., 2003b), which reports on neural progenitor specification during differentiation, and monitored GFP expression across a time course of neural differentiation under defined conditions (Ying et al., 2003b), with or without a pharmacological block to Erk activation (PD0325901, hereafter referred to as Meki) (Bain et al., 2007). Sox1-GFP is normally expressed from 48 hr after the initiation of differentiation. Surprisingly, we observed little or no change in GFP induction as a result of Meki treatment, with only a small and insignificant (Student's *t* test, *p* > 0.05) difference in the percentage of cells expressing Sox1-GFP between days 3 and 5 (Figure 1A). We confirmed this observation in the wild-type (WT) ESC line E14Ju (Hamilton et al., 2013), in which we saw a robust induction of SOX1 protein at day 4 that coincided with an almost complete downregulation of the ESC marker OCT4 (Figure 1B). A time course of gene expression analysis across the first 4 days of differentiation showed that the induction of the master regulator of neural specification, *Zfp521* (Kamiya et al., 2011), was unchanged (Figure 1C) when Erk activ-

ity was uniformly inhibited by more than 85%  $\pm$  3.5% (Figures S1A and S1B). Similar to previously reported work (Jaeger et al., 2011), we saw an upregulation in the ventral midbrain marker *Lmx1a* (Chung et al., 2009) (Figure 1C), indicating that the neural progenitors are capable of responding to Meki. Analysis of gene expression at day 7 of the protocol showed normal induction of a range of neural markers (Figure 1D), as well as expression of morphologically distinct,  $\beta$ III TUBULIN-expressing neurons (Figure 1E). Consistent with previously published work (Lowell et al., 2006), we saw the persistence of some ES-like cells at day 7 of the assay, with a slightly higher percentage persisting in Meki-treated cultures, as judged by flow cytometry for the cell-surface marker PECAM1 (Figure 1F). We next looked at how ESC-associated gene expression changed in our system. Under self-renewal conditions, inhibition of Erk activity resulted in a significant increase in the expression of many ESC markers such as *Nanog*, *Tfcp2l1* (Martello et al., 2013), and *Tbx3* (Niwa et al., 2009) (Figure 1G); however, this difference was lost within approximately 24–48 hr after differentiation was induced. Thus, although we observed increased pluripotent gene expression in response to Meki, differentiation toward neural progenitors was essentially normal.

We considered whether the lack of efficient inhibition of neural differentiation was due to the presence of an Erk-dependent epiblast “primed” population of cells in standard LIF/serum culture that was able to directly differentiate toward neural lineages, thus circumventing the Erk requirement (Jaeger et al., 2011). To eliminate this epiblast-primed population, we precultured cells in “ground-state” ESC culture conditions (Ying et al., 2008). These conditions, known as 2i, contain both Meki and a Gsk-3 $\beta$  inhibitor, Chiron. Preculture in 2i neither inhibited neural differentiation nor resulted in Erk dependence for Sox1 induction (Figure 1H). To determine whether this phenomenon was restricted to a specific ESC genetic background, we compared the ability of several ESC lines to form neural precursors in the absence of Erk activity, all from preculture in 2i (Figure S1D), and found that Erk-independent neural specification was consistent between 129Ola, C57B6, and 129Ola:C57B6<sup>F1</sup> ESCs. In fact, we found that C57B6 ESCs could only form neural tissue in the presence of Meki. Furthermore, we tested different sources of basal media, with and without retinol (which is metabolized to retinoic acid, a potent inducer of neural identity [Figure S1C]), two batches of serum, and a variety of sources of Meki (Figure S1C; data not shown). In all cases, we observed neural induction in the presence of Meki. Thus, it appears that inhibition of Mek-Erk signaling does not block neural differentiation, although it supports epiblast/pluripotent gene expression in the presence of LIF.

The Mek inhibitor PD0325901 is the most specific Mek inhibitor described to date, but it loses specificity for Mek1/2 isoforms at concentrations greater than 1  $\mu$ M (Bain et al., 2007). Although 1  $\mu$ M Meki is sufficient to block acute stimulation with FGF4 (Figure S2A), to ensure Meki was able to completely suppress Mek1/2 activity at a dose where it would not affect other Mek isoforms, we used a combinatorial chemical-genetic approach where the inhibitory effect of Meki was augmented through genetic depletion of Erk2, the main Erk isozyme expressed in ESCs (Hamilton et al., 2013). We found that Erk2 mutant ESCs



**Figure 1. Neural Differentiation Can Proceed in the Presence of a Pharmacological Block to Mek-Erk Signaling**

(A) Flow cytometry measuring the expression of the neural progenitor reporter Sox1-EGFP in 46C cells treated with either Meki (PD0325901) at 1 μM or DMSO across a time course of neural differentiation. A slight delay in Sox-GFP induction is observed on days 3–5, whereas cultures are largely indistinguishable with respect to their Sox1 expression by day 6.

(B) Immunofluorescence analysis of day 4 neural differentiation of the ESC line E14Ju confirming the presence of SOX1 (green) protein in both conditions. DNA is counterstained with DAPI, and cells were also stained for OCT4 (red).

(legend continued on next page)



exhibited a dramatic increase in drug sensitivity compared to WT ESCs, with a 50% reduction in the  $IC_{50}$  for PD0325901 (WT: 60 nM, Erk2<sup>-/-</sup>:30 nM), which correlated with a decrease in the phosphorylation of the Erk1/2 substrate p90Rsk (Figures S2B and S2C). We therefore analyzed the phenotype of these Erk2 mutant ESCs treated with varying concentrations of Meki in both self-renewing and differentiating conditions. Consistent with our previous observations, even with this heightened sensitivity, Meki was unable to block neural differentiation (Figure 2A), with expression of neural markers *Ngn2* and *Nestin* (Couillard-Despres et al., 2008) being similar between genotypes and conditions (Figure 2B). We did however note an inverse correlation between *Nanog* expression under self-renewing conditions and levels of Erk phosphorylation, such that in Erk2 mutants, *Nanog* expression plateaued at 0.3  $\mu$ M Meki (Figure 2B), and this was also the point where we observed saturating inhibition of Erk activity (Figure 2C; Figure S2A). Based on the capacity of Erk2<sup>-/-</sup> ESCs to differentiate normally in concentrations of Meki that completely block all Erk phosphorylation, we conclude that Erk activation is not required for progression in neural differentiation.

### Erk Signaling Is Required for PrE Differentiation

In vivo, Fgf4 is the major determinant of the segregation of epiblast and PrE. Fgf signaling is also required for the conversion of ESCs to extra embryonic endodermal stem (XEN) cell lines (Cho et al., 2012), but it is unclear whether this requirement occurs at the level of Erk signaling or in other pathways downstream of Fgf. Moreover, because we found that Erk signaling was not required for epiblast or neural specification, we thought it was important to test the requirement for Erk signaling in PrE differentiation from ESCs and determine whether it is required at the level of cell specification or expansion. We induced cells to differentiate by LIF withdrawal in either monolayer culture (Figures 3A and 3B), or as 3D aggregates (Figures 3C–3E), both of which allow PrE differentiation (Canham et al., 2010; Morgani et al., 2013). We found that Erk inhibition led to a robust block to endodermal differentiation. Antibody staining showed that Meki inhibited the expression of the PrE marker *Gata6* in these cultures, while sustaining *Oct4* expression (Figure 3A). Figure 3B shows flow cytometry assessing induction of the early endodermal marker PDGFRA alongside the ESC marker PECAM1 (Rugg-Gunn et al., 2012), following 5 days of differentiation in the absence of LIF. Meki completely blocked this differentiation resulting in a homogenous population of PECAM1-positive PDGFRA-negative ES-like cells. Importantly, we noted no difference in the number of cells in these cultures, suggesting this phenotype is not a result of selective cell survival. Moreover, a similar block to differentiation was observed when cells were

cultured as hanging-drop embryoid bodies, and then allowed to adhere and outgrow. This method gives a high proportion of PrE cells (PDGFRA-positive, *GATA6*-positive) that was completely lost when cells were differentiated in the presence of Meki (Figures 3C and 3D). Real-time quantitative PCR (qPCR) shows that ESC markers *Oct4*, *Sox2*, and *Nanog* were sustained in the presence of Meki, whereas the normal induction of the PrE markers *Pdgrfa*, *Gata6*, *Sox7*, and *Sox17* (Artus et al., 2011) was inhibited (Figure 3E). Taken together, these data demonstrate an absolute requirement for Erk activation in PrE specification.

### Erk Induction Promotes PrE by Suppressing Epiblast Identity in ESCs

We investigated the transcriptional response to Erk in ESCs by engineering an inducible gain-of-function model. We generated a constitutively active form of c-Raf, an effector kinase upstream of Mek-Erk, which lacked the Erk-dependent repressive domains (c-Raf<sup>A26–303</sup>) (Samuels et al., 1993) and fused it to two different drug-inducible peptides. At the amino terminus, we introduced an FKBP<sup>L106P</sup> tag (Banaszynski et al., 2006), that targets the protein for degradation in absence of the small molecule, Shd1. At the carboxyl terminus, we fused a variant of the ligand binding domain of the estrogen receptor that binds selectively to 4-hydroxy tamoxifen (4OHT). The resulting fusion protein (FCXC<sup>T2</sup>) (Figure 4A) enabled the induction of Erk phosphorylation in response to 4OHT and Shd1. To ensure that the major source of Erk signaling in this system was derived from the exogenous FCXC<sup>T2</sup>, cells were cultured in the presence of a small molecule inhibitor of the Fgf receptor PD173074 (Fgfri), for 24 hr prior to induction, alongside Shd1 to stabilize the fusion protein, with both drugs present during the time course of 4OHT stimulation. Given that Erk signaling is required for PrE specification in the preimplantation mammalian embryo from which ESCs are derived (Nichols et al., 2009), we introduced the FCXC<sup>T2</sup> construct into the Hhex-venus (HV) knockin line that has been shown to be a sensitive reporter for PrE priming and specification (Canham et al., 2010; Morgani et al., 2013). This cell line enables the isolation of ESCs that are primed (biased) toward, but not yet committed to, PrE as well as providing a sensitive reporter for PrE differentiation. Following stabilization and induction of FCXC<sup>T2</sup>, a rapid and sustained increase in pErk was detected that could be blocked if induction was performed in the presence of Meki (Figure 4B). To ensure that FCXC<sup>T2</sup> activity was not affecting parallel signaling pathways, we analyzed the phosphorylation status of components of the LIF-STAT3, Akt, Gsk3- $\beta$ -catenin, and BMP-SMAD pathways and found only minimal sporadic changes in

(C) Real-time qPCR assays showing the expression of early neural (*Zfp521*) and ventral midbrain marker (*Lmx1a*) at the indicated times during neural differentiation.

(D) Real-time qPCR assays showing the expression of the indicated neural markers following 7 days of neural differentiation.

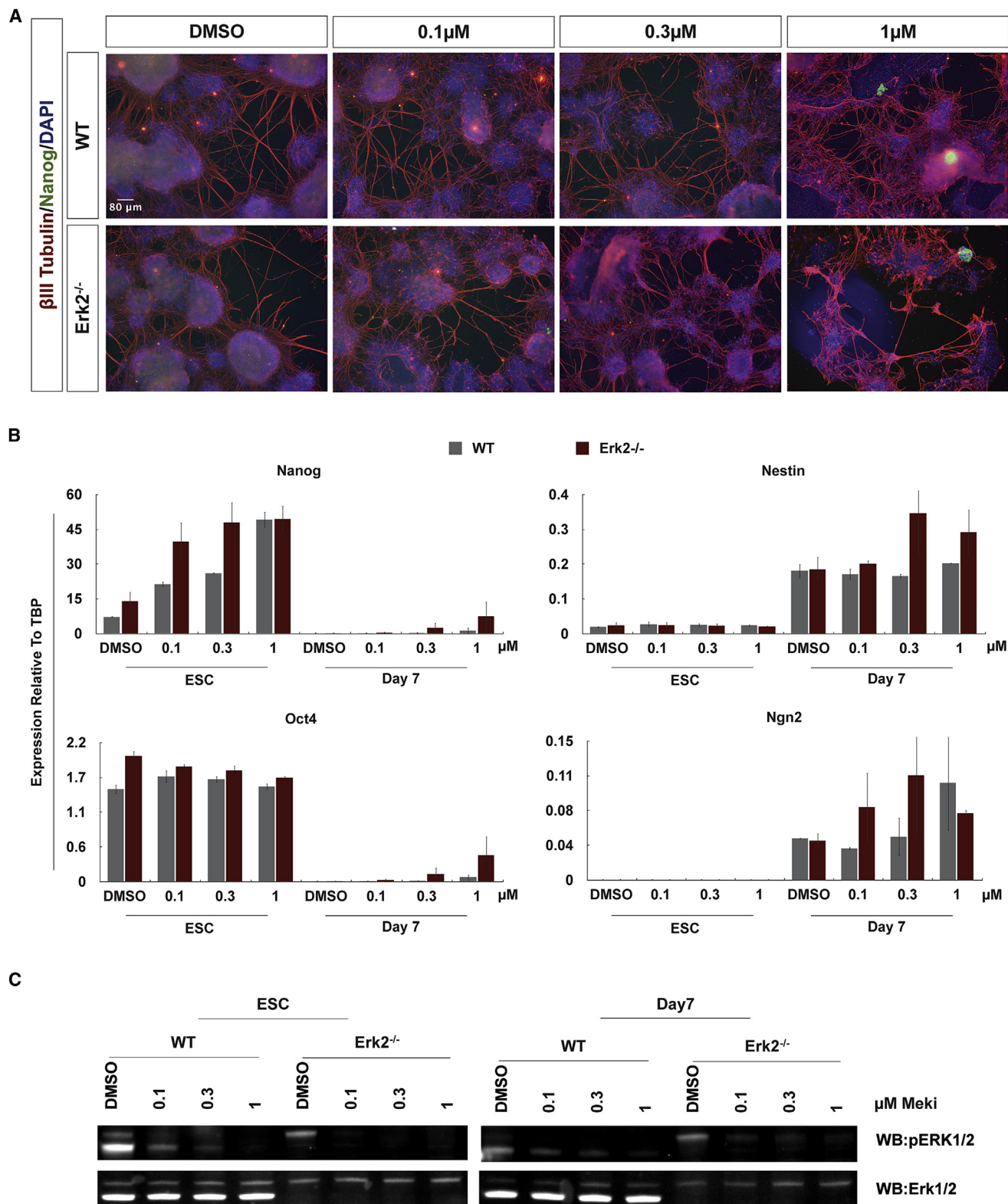
(E) Immunofluorescence analysis of day 7 neural differentiation of ESC line E14Ju for the neural marker  $\beta$ III tubulin (yellow) and the ESC marker OCT4 (red). DNA is counterstained with DAPI (purple).

(F) Flow cytometry reporting on the presence of ESCs (PECAM1-positive) following 7 days of neural differentiation, DMSO <1%, Meki ~4% PECAM1 positive.

(G) Real-time qPCR analysis for the expression of various ESC markers at the indicated times.

(H) Flow cytometry for Sox1-GFP expression following neural differentiation from cells grown in either serum-free “2i” or standard serum:LIF culture conditions; no difference was observed in the extent of Sox1-GFP induction.

All data are presented as the mean  $\pm$  SD of independent experiments, except for Figure 1D, which is the mean  $\pm$  SD of parallel experiments. See also Figure S1.



**Figure 2. Acute Attenuation of Erk Activity in  $Erk2^{-/-}$  ESCs Fails to Inhibit Neural Differentiation**

(A) Immunofluorescence for  $\beta$ III-TUBULIN (red) and NANOG (green) following 7 days of neural differentiation of the  $Erk2^{-/-}$  or E14Ju parental-control ESCs in the presence of the indicated concentration of Meki. Cultures were largely identical except for a slight decrease in overall numbers of  $Erk2$  knockout cells present in cultures treated with 1  $\mu$ M Meki. DNA is counterstained with DAPI.

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phosphorylation of these components despite 24 hr of sustained Raf-Erk induction (Figure 4C). Furthermore, following FCXC<sup>T2</sup> induction, Erk activity was detected in both the nucleus and the cytoplasm, which could again be blocked by Meki (Figure 4D).

Next, we analyzed PrE priming in response to Erk by monitoring *Hhex* RNA and HV expression. Flow cytometry for venus fluorescence and real-time qPCR for *Hhex* expression showed a dramatic increase in promoter activity following 24 hr of FCXC<sup>T2</sup>/Erk induction. This induction was also blocked by cotreatment with Meki (Figures 4E and 4F). Although Erk activity has been reported to be dispensable for ESC proliferation (Ying et al., 2008), we saw a decrease in the percentage of cells in S phase when Erk activity was inhibited by Fgfri, which could be rescued by activation of downstream c-Raf/Erk (Figure 4G). Because the efficiency of self-renewal (Coronado et al., 2013) and lineage potential (Calder et al., 2013) have been linked to cell-cycle position, we asked whether Erk-dependent progression into S phase might stimulate *Hhex* expression. When cells were sorted based on their DNA content/cell-cycle position and the magnitude of HV induction was measured in each cell-cycle position, we did not observe any cell-cycle-dependent differences in the magnitude of venus fluorescence (Figure S3A). Taken together, we conclude that Erk induction can stimulate G1/S progression and PrE differentiation, but these events do not appear to be linked.

To understand the transcriptional mechanisms downstream of Erk induction, we performed a time course of microarray expression analysis across the first 24 hr of Erk induction (Figure 5A) (GEO accession number GSE59755). The earliest genes induced (60 min) were the canonical FGF-Erk immediate early genes (IEG) *cFos* and *Egr1* (Hamilton et al., 2013). The transcriptional response increased over time, and by 24 hr the expression of over 13,000 genes had changed by at least 2-fold (Table S1).

We have described several functionally distinct subpopulations in self-renewing ESC cultures based on the differential expression of HV and the cell-surface marker, SSEA1 (Canham et al., 2010; Morgani et al., 2013) (Figure 5B). Coexpression of HV and SSEA1 (HV<sup>+</sup>S<sup>+</sup>) marks a PrE-primed self-renewing subpopulation of ESCs. Cells in this population exhibit a PrE bias in differentiation but self-renew in standard ESC conditions. HV<sup>+</sup>S<sup>−</sup> cells are committed to PrE differentiation. HV<sup>−</sup> populations (both SSEA1<sup>+/−</sup>) represent ESCs primed to differentiate toward epiblast lineages, such as neural. We have previously defined gene sets enriched in these populations, depicted in Figure 4B (Canham et al., 2010), and we assessed their behavior in our Erk induction time course data set (Figure 5C). We observed a striking overlap between the genes that were enriched in both HV<sup>+</sup> populations and those induced by Erk activity. Moreover, genes enriched in the HV<sup>−</sup> populations were repressed by Erk induction. In addition to the overlap of gene expression data sets observed, we found that genes enriched in the HV/SSEA1

double-positive, PrE-primed population were induced earlier (beginning at 240 min) than those enriched in the HV-positive, SSEA1-negative, PrE differentiated population, indicating that our Erk induction data set captures the transcriptional hierarchy of PrE priming and differentiation. Because activation of FgfR2 signaling had been shown to repress *Nanog* expression (Santostefano et al., 2012), we asked how an extended list of ESC markers, defined as having an ESC restricted expression pattern (Mitsui et al., 2003), responded to Erk induction (Figure 5D). We observed early repression of many genes whose expression has been reported to be heterogeneous in ESCs, and whose forced overexpression supports LIF-independent self-renewal (Chambers et al., 2003; Festuccia et al., 2012; Martello et al., 2013; Mitsui et al., 2003; Niwa et al., 2009). In summary, the use of our inducible model shows Erk to be the master regulator of PrE priming and differentiation. The difference between these cell states is determined by the duration of Erk activation.

### Erk Targets *Nanog* to Derepress *Gata6*

In the blastocyst, the time preceding the segregation of epiblast and PrE lineages is marked by a period of transcriptional heterogeneity, with differential and overlapping expression of either *Nanog* or *Gata6* (Chazaud et al., 2006; Frankenberg et al., 2011; Guo et al., 2010). Mutual antagonism between these two transcription factors is thought to be a mechanism of lineage segregation (Mitsui et al., 2003; Singh et al., 2007). Because of the high degree of temporal resolution in our data set, we were able to ask whether Erk activity directly regulated the expression of *Gata6* or *Nanog*. We observed repression of both *Nanog* mRNA and protein (Figures 6A and 6B) occurred at 4 hr, which preceded the induction of *Gata6* between 8 and 24 hr (Figure 6C). We then asked whether the repression of the *Nanog* promoter was direct. To do this, we induced Erk activity in the presence or absence of the translational inhibitor cycloheximide and observed that *Nanog* expression was repressed following 6 hr of FCXC<sup>T2</sup> induction regardless of whether there was new protein synthesis or not (Figure 6D). Moreover, we used formaldehyde-assisted isolation of regulatory elements (FAIREs) (Giresi et al., 2007) to determine whether Erk activation altered the extent of nucleosome free DNA in the *Nanog* locus and found the region to be condensed following 24 hr of Erk stimulation (Figure S4A).

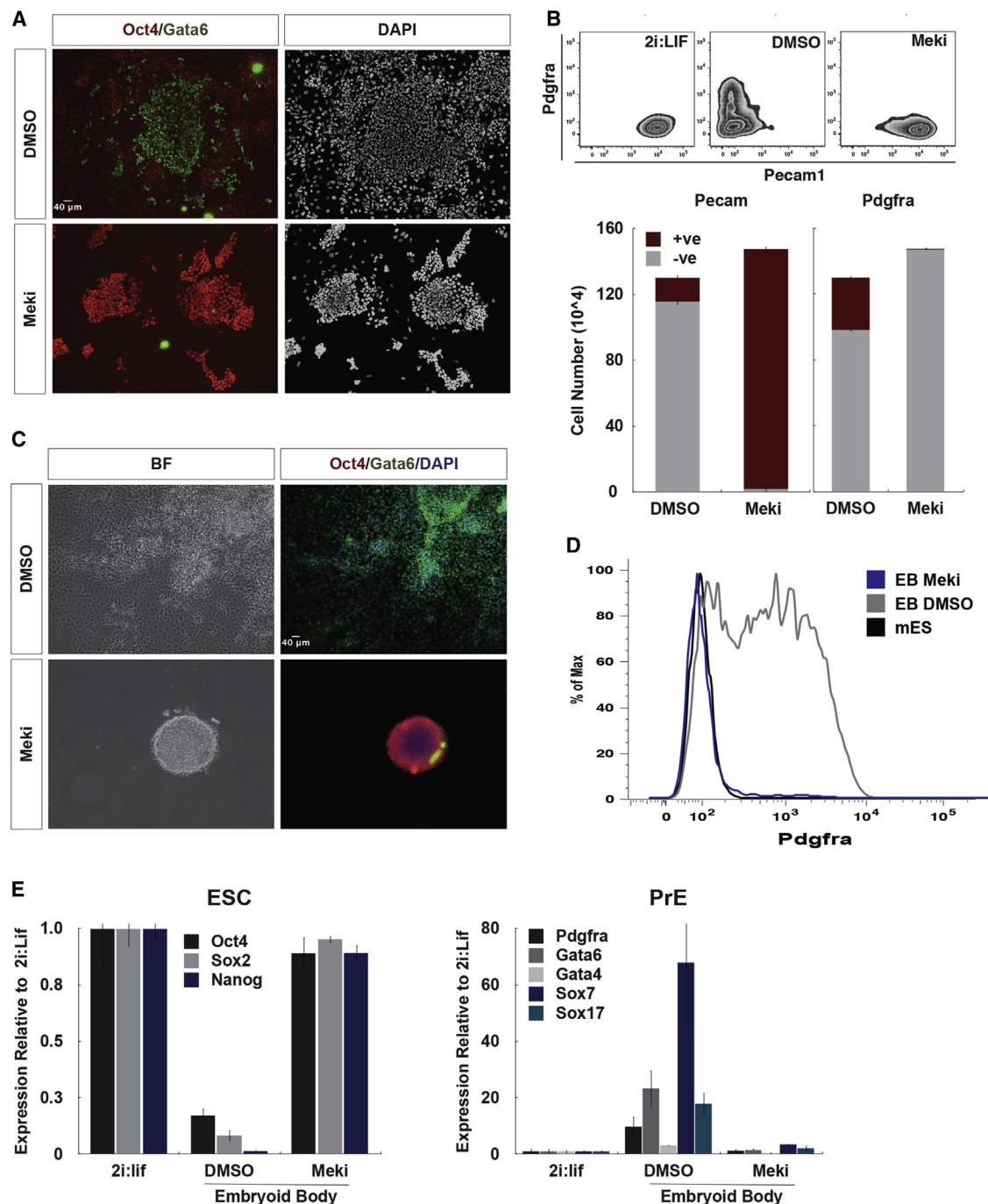
Because our data suggested that suppression of *Nanog* preceded any detectable increase in *Gata6* expression, we asked whether forced expression of *Nanog* could block Erk-dependent *Gata6* induction. We transiently transfected cells with a *Nanog*-expressing plasmid, allowed 24 hr for protein synthesis, and then induced FCXC<sup>T2</sup> activity for a further 24 hr (Figure 6E). We observed a reduction in Erk-dependent *Gata6* expression (Student's *t* test >0.008), when *Nanog* was exogenously expressed (Figure 6F); however, the forced expression of *Nanog* was unable to suppress the Erk-dependent repression of a range of epiblast/pluripotency markers (Figure 6G), even the direct *Nanog* targets

(B) Real-time qPCR analysis for both ESC (*Nanog* and *Oct4*) and neural marker (*Nestin* and *Ngn2*) expression in WT and Erk2 knockout cells. Data are presented as the mean  $\pm$  SD of independent experiments.

(C) Western blot for phospho-Erk1/2 (pTEpY) before and after neural differentiation. Bands from separate gels were excised and transferred to the same membrane and are therefore comparable. No phospho-Erk1 could be detected in Erk2 mutant cells treated with 1  $\mu$ M Meki.

See also Figure S2.





**Figure 3. Erk Signaling Is Required for PrE Specification**

(A) Immunofluorescence images showing the persistence of a largely homogenous OCT4 (red) -positive, GATA6-negative (green), population of cells following 5 days of differentiation in the presence of Meki, in contrast to DMSO-treated cultures.

(B) Flow cytometry for expression of the ESC marker PECAM1, and the early endodermal marker PDGFRA at day 0 (2i:LIF), and following 5 days of LIF withdrawal. In this context, inhibition of Erk activity did not result in a decrease in the number of cells present at the end of the assay, but a complete block to Pdgfra induction. Quantitation of the flow cytometry is presented beneath the scatterplots. Data are presented in relative to total cell number.

(C) Immunofluorescence analysis of adherent embryoid bodies following 6 days of differentiation showing almost homogeneous GATA6 (green) induction under control conditions. In the presence of Meki, only OCT4 (red) -positive, GATA6-negative cells persists.

(D) Flow cytometry showing a complete block in PDGFRA expression in cultures treated with Meki.

(E) Real-time qPCR analysis for ESC and PrE markers following 6 days of embryoid body differentiation in the presence or absence of Meki. Data are normalized to 2i:LIF and presented as the mean  $\pm$  SD of independent experiments.

*Esrrb* and *Klf4* (Festuccia et al., 2012). To determine whether the persistence of Gata6 induction observed under these conditions was an artifact of the heterogeneous nature of transfection assays, we generated a Nanog-mCherry fusion protein and sorted cells that expressed low, physiological levels of NANOG from transfected populations following FCXC<sup>T2</sup> induction (Figures S4B and S4C). Analysis of endogenous *Nanog* and *Esrrb* expression showed, again, identical levels of repression even in the presence of exogenous NANOG-mCHERRY; however, the extent to which Gata6 induction was blocked was now considerably more pronounced (Figures 6H–6J). Altogether, our data suggest a model whereby Erk acts directly on the epiblast/pluripotency network to inhibit transcription, but Nanog itself is sufficient to block PrE specification through Gata6 repression.

## DISCUSSION

In this paper, we have shown that Erk signaling is required for PrE differentiation and specifically acts to repress self-renewal as a means to promote differentiation toward this lineage (Figure 7). Time-limited induction of Erk signaling stimulates PrE priming in ESC culture including the downregulation of epiblast/pluripotency markers. Although these events are Nanog independent, the normal induction of PrE gene expression (e.g., Gata6) is sensitive to Nanog levels. Conversely, neural specification proceeds normally in the absence of detectable Erk activity suggesting that the enhanced epiblast/pluripotent gene expression observed in absence of Erk activity has little impact on epiblast transition and neural differentiation.

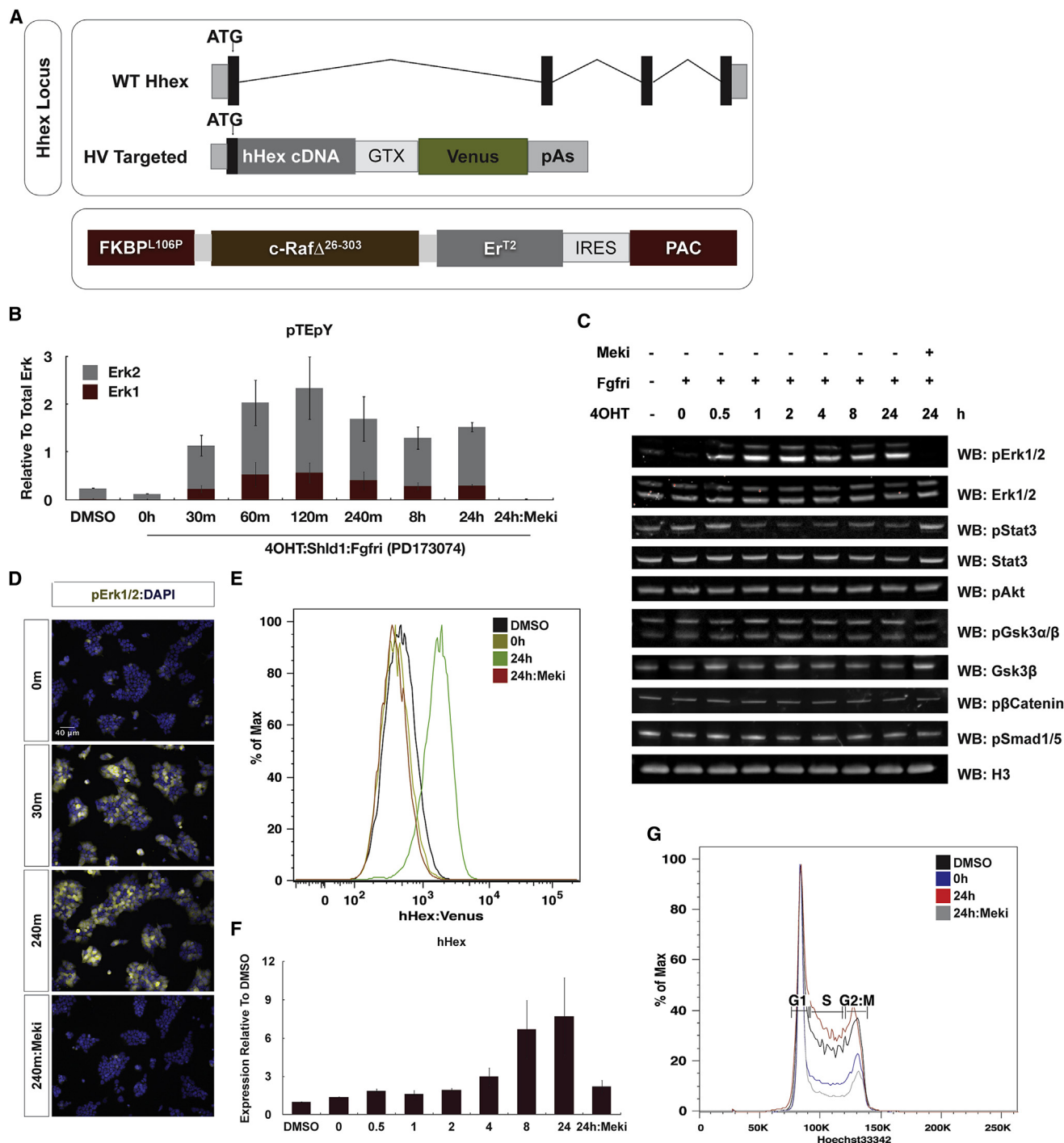
Because Erk inhibition resulted in enhanced levels of the ESC gene regulatory network (GRN), why does this specifically inhibit endodermal, but not neural differentiation? Perhaps this is because Erk signaling is fundamentally required for differentiation of the mesoderm and endoderm lineages. In vivo, at the stage from which ESCs are derived, the relevant mesendoderm lineage is PrE and Erk regulates the binary choice between epiblast and PrE. As a consequence, Erk activity in ESC cultures is associated with downregulating the pluripotency network as part of the PrE differentiation program. In cells that have already made the choice to become epiblast, the differentiation program may involve new factors, such as TFE3, that are independent of the MAP kinase pathway (Betschinger et al., 2013). Additionally, inhibitors of Wnt signaling act to downregulate the epiblast network during differentiation toward gastrulation stage lineages. Our data suggest that the Erk-regulated ESC GRN functions largely in blocking endodermal differentiation and that self-renewal is maintained through repressing predefined lineage specific programs. Naive pluripotency would therefore be supported by a combined block to PrE differentiation, through the inhibition of Erk signaling, and mature ectoderm as a consequence of Wnt stimulation. Consistent with this observation is our recent finding that ESCs lacking Groucho/TLE corepressors, antagonist of the Wnt pathway, are unable to progress efficiently in neural differentiation (Laing et al., 2014).

The requirement for Fgf signaling via Erk activity in the earliest steps of differentiation toward a primed, egg cylinder-like epiblast provided a phased model for neural differentiation that required

Erk stimulation and BMP antagonism (Ying et al., 2003a). Our data indicate that Erk signaling is not required for differentiation of naive epiblast into neural progenitors. Consistent with our observations is the ability of Erk2 mutant ESCs to develop past epiblast stage in tetraploid chimera experiments with wild-type extraembryonic tissue (Voisin et al., 2010). What then explains the requirement for Fgf signaling? Perhaps a role in neural cell viability manifested itself as induction in earlier studies. Thus, although Fgf signaling is not necessary for neural induction, it could be required for cell and this would not have been detected in previously reported population analyses, primarily flow cytometry and real-time PCR. These observations are consistent with a requirement for Fgf signaling in the survival of early telencephalic neurons (Paek et al., 2009), but not in neuroectodermal specification (Di-Gregorio et al., 2007). In addition, signaling downstream of FGF receptors activates the Akt pathway (Ong et al., 2001), which has been shown to regulate neuronal cell survival (Zhang et al., 2013). It is also possible that both the antagonism of BMP signaling and Erk activation feed into similar regulatory programs. A major activity downstream of Erk is the promotion of epithelial to mesenchymal transition (EMT) (Oki et al., 2010). It was recently demonstrated that BMP signaling acts through Id proteins to inhibit neural differentiation in ESCs by suppressing an EMT-like event (Malaguti et al., 2013). Moreover, a component of the mechanism by which Oct4 suppresses differentiation is via sustained BMP expression and the maintenance of adherens junctions and E-cadherin expression (Livigni et al., 2013). Thus, it may be that Erk activity is required for neural induction in contexts where inhibition of BMP signaling is not sufficient to promote the downregulation of E-cadherin on its own. It is intriguing to note that in the mouse, where BMP antagonism appears sufficient to induce neural differentiation, there is no evidence for significant Erk activation in pregastrulation epiblast (Corson et al., 2003), whereas in chick, where early neural differentiation is Erk-dependent, activated Erk is detected (Lunn et al., 2007).

ESC cultures contain populations of primed epiblast and PrE cells, and we found that Erk activity suppressed self-renewal to promote PrE priming. In ESC cultures, this priming is dynamic, and we observed that the set of early Erk responding genes in our data set corresponded to genes expressed in primed, but not differentiated populations of ESCs. Perhaps this means that the existence of primed states in ESC is a product of the negative-feedback loops normally restricting the duration of Erk signaling in ESCs.

In vivo, prior to lineage segregation, blastomeres transiently coexpress both Gata6 and Nanog. Loss of Fgf4 does not eliminate the early expression of Gata6 (Kang et al., 2013) but results in a failure of cells to downregulate Nanog and segregate endoderm identity. Thus, it may be that Fgf:Erk signaling is essential to downregulate pluripotency/epiblast-associated genes such as Nanog, relieving repression of the endodermal GRN and allowing lineage resolution. In vitro, we found that Erk induction resulted in suppression of the epiblast/pluripotency network prior to the induction of Gata6 expression. It therefore seems likely that the major function of this signaling pathway is to suppress epiblast gene transcription thereby enabling the stable expression of Gata6. Because Nanog overexpression can inhibit PrE differentiation, but not downregulation of the epiblast GRN in



**Figure 4. A Heterologous System for Drug-Inducible Erk Activation**

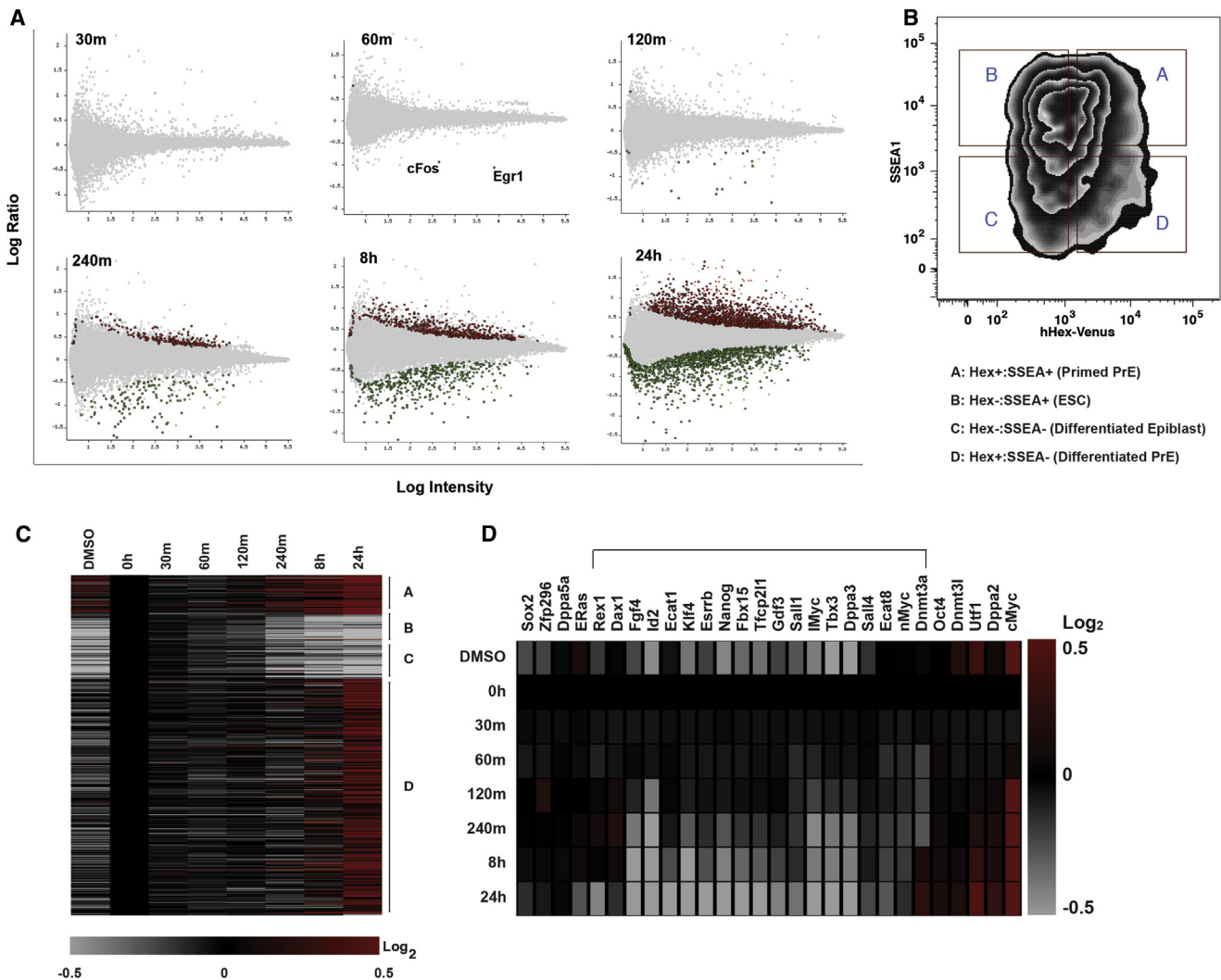
(A) A schematic illustration of the inducible system. The c-Raf fusion protein (FKBP<sup>L106P</sup>-cRaf-Δ-cRaf-Er<sup>T2</sup>:FCXC<sup>T2</sup>) contains an N-terminal FKBP and C-terminal mutant estrogen receptor (ERT2) and is constitutively expressed from the CAG promoter in HV ESCs. The Hhex reporter contains a reiterated Gtx IRES element and intact Hhex cDNA inserted into the first exon of the Hhex genomic locus.

(B) Western blot quantification of Erk phosphorylation following FCXC<sup>T2</sup> induction for different periods of time. Data are presented as the mean ± SD of independent clones.

(C) Western blot analysis for the phosphorylation status of components of several signaling pathways known to be active in ESCs: Erk1/2 (T<sup>183</sup>:Y<sup>185</sup>), STAT3 (T<sup>705</sup>), Akt (S<sup>473</sup>), Gsk3α/β (S<sup>21/9</sup>), β-catenin (S<sup>33/37</sup>:T<sup>41</sup>), and SMAD1/5 (S<sup>463/465</sup>), showing only minor fluctuation in their activity across the time analyzed.

(D) Immunofluorescence for pErk1/2 showing a heterogeneous mix of nuclear and cytoplasmic active Erk following FCXC<sup>T2</sup> induction.

(legend continued on next page)



**Figure 5. Erk Induction Promotes PrE by Suppressing Epiblast Identity in ESCs**

(A) Microarray time course for gene expression induced by FCXC<sup>T2</sup> induction. Pairwise comparison (false discovery rate >0.05%, >2-fold expression change, average of independent clones) between 0 hr and the indicated time points. The earliest genes to be induced were the canonical MAPK targets *cFos* and *Egr1* at 60 min.

(B) Flow cytometry analysis for HV and SSEA1 showing the sorting strategy used in Canham et al. (2010) to identify populations of primed and differentiating PrE for expression analysis.

(C) A heatmap showing the correlation between Erk regulated targets and their enrichment in each of the indicated populations from Canham et al. (2010). The expression pattern of genes that were enriched in each population (>2-fold average of two clones) was analyzed in the FCXC<sup>T2</sup> data set and presented as fold change relative to 0 hr. We observed that genes enriched in both HV-positive populations were induced by Erk activity, whereas genes in the HV negative populations were repressed. Genes enriched in the SSEA1:HV-positive population (A) PrE-primed population were repressed by Fgfri treatment (0 hr) and were induced early, starting at 240 min. Genes in population (D), which represents differentiating PrE, were induced later at around 8 hr.

(D) A heatmap showing the transcription response of several ESC-associated transcripts (ECATs) including *Oct4* and *Sox2* in response to Erk activation. The majority of genes reported to be heterogeneously expressed in ESCs are indicated by the bracket and were rapidly repressed upon FCXC<sup>T2</sup> induction.

response to Erk, it suggests that individual epiblast transcription factors are able to directly repress PrE gene transcription, but not sustain the GRN itself.

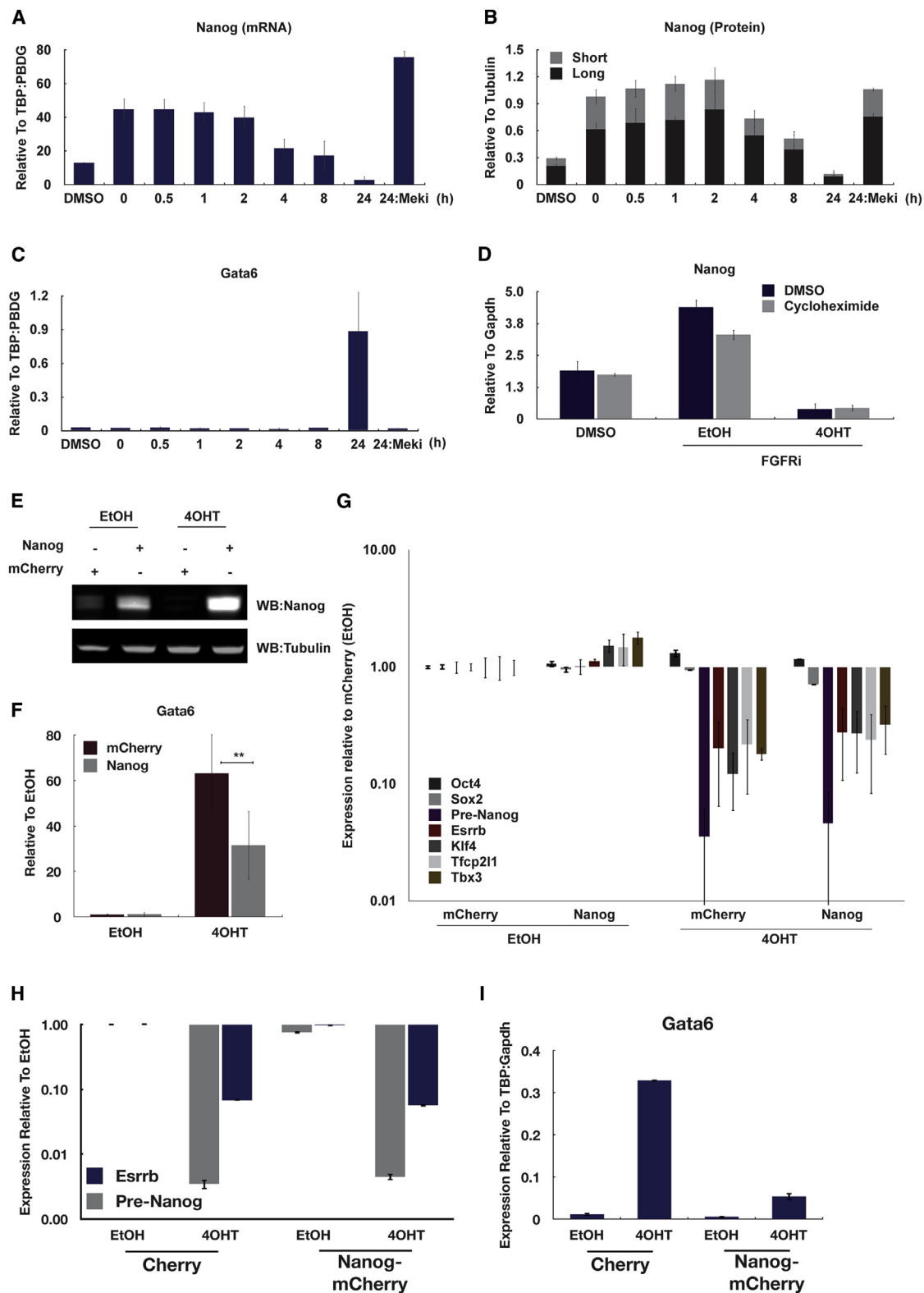
Erk was recently linked to the induction of developmental genes via the recruitment of PRC2 and phosphorylation of Ser5 in the RNA polymerase II CTD (Tee et al., 2014). This

(E) Cell-cycle analysis showing an Erk-dependent block in G1 progression. Cells were treated as indicated, stained with Hoechst 33342, and analyzed by flow cytometry.

(F and G) FCXC<sup>T2</sup> induction results in an increase in *Hhex* promoter activity as judged by venus fluorescence (F) and real-time qPCR (G). Data are presented as the mean  $\pm$  SD of independent clones.

See also Figure S3.



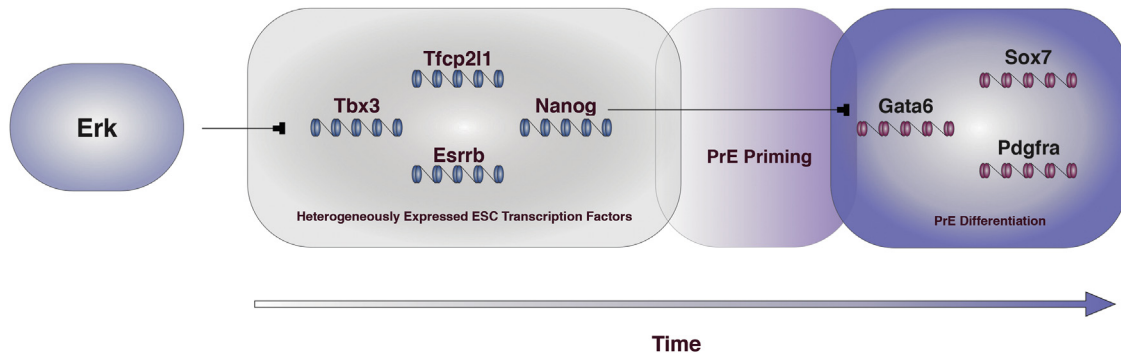


**Figure 6. Nanog Expression Inhibits Gata6 Induction but Is Unable to Counteract Erk-Mediated Suppression of Pluripotency**

(A) Real-time qPCR showing *Nanog* mRNA levels in response to FCXC<sup>T2</sup> induction.

(B) Western blot quantification showing NANOG protein levels in response to the same treatments as in (A).

(legend continued on next page)



**Figure 7. A Cartoon Illustrating the Role of Erk Signaling in ESC Self-Renewal and Differentiation**

Short-term stimulation of Erk signaling results in a cell state transcriptionally similar PrE-primed ESCs. This state is reversible in standard ESC culture, but when cells are challenged to differentiate they show a bias toward PrE and away from epiblast differentiation. This short-duration stimulation of Erk results in the direct inhibition of the expression of transcription factors in the epiblast/pluripotency network known to be heterogeneously expressed in ESC, whereas prolonged stimulation of Erk results in PrE differentiation and the upregulation of Gata6. Nanog is able to block this induction of Gata6, but its expression is insufficient to counteract the inhibition of the pluripotent/epiblast gene expression in response to Erk activation.

conformation is believed to pause RNA polymerase, and perhaps this regulatory mechanism is relevant to the suppression of the epiblast/pluripotency network in response to Erk activation. In this case, activation of Erk could lead to PRC2 recruitment and transcriptional pausing as a first step in the downregulation of epiblast genes as cells begin to prime for PrE differentiation. This suggests a permissive rather than inductive paradigm for Erk-mediated lineage specification, whereby Erk activity acts as a licensing factor, derepressing endoderm fate by directly repressing epiblast identity.

## EXPERIMENTAL PROCEDURES

### ESC Culture

ESC lines (Table S2) were maintained in complete ESC medium: GMEM (Sigma-Aldrich) supplemented with 10% FBS (Gibco), 100  $\mu$ M 2-mercaptoethanol (Sigma-Aldrich), 1  $\times$  MEM nonessential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate (all from Gibco), and 1,000 units/ml LIF (made in house) on gelatinized tissue culture flasks (Corning). For serum-free culture, ESC lines were maintained in N2B27 (made in house) or from STEMCELL Technologies, supplemented with 3  $\mu$ M Gsk3i (Chir99021: Axon Medchem, or Stemgent), 1  $\mu$ M Mek1 (PD0325901: Sigma-Aldrich, Stemgent, or Axon Medchem) and LIF, and passaged with Accutase (Sigma-Aldrich). The FGFR inhibitor PD173074 was used at 250 nM and was from Sigma-Aldrich. Recombinant mouse Fgf4 was from R&D Systems

and was used at 20 nM. Neural monolayer differentiation was performed as outlined by Ying et al. (2003b). In brief, cells were collected, washed in N2B27, and seeded at a density of  $10^4/\text{cm}^2$ , with daily media changes. To differentiate ESCs toward endodermal lineages, cells were plated in complete, serum-containing, ES medium without LIF, at a density of  $0.5 \times 10^4/\text{cm}^2$ . Embryoid body differentiation was performed by suspending cells at a density of  $1.2 \times 10^4/\text{ml}$  in 30  $\mu$ l drops for 48 hr and then allowing spheres to adhere to gelatin coated dishes and expand for a further 4 days. 4-OHT (Sigma-Aldrich) was used at 250 nM and Shld1 (Clontech Laboratories) was used at 500 nM. Nanog (ENSMUST00000012540) was cloned from E14Ju cDNA into the pPyCAG-IP vector (Chambers et al., 2003) by standard methods. Transient transfection was performed using Lipofectamine 2000 (Life Technologies).

### Real-Time qPCR

Total RNA was collected using either Trizol (Invitrogen) or the RNeasy Mini Kit (QIAGEN). Genomic DNA was eliminated by DNase treatment (QIAGEN), and 1  $\mu$ g of total RNA was used for first-strand synthesis with SuperScript III reverse transcriptase according to the manufacturer's instructions. cDNA corresponding to 10 ng total RNA was used for real-time qPCR analysis using the Roche LC480, and target amplification was detected with the Universal Probe Library system. See Table S3 for a list of primers and probes used.

### Western Blotting

Blotting was performed as previously described (Hamilton et al., 2013) except that primary antibodies were detected using fluorescently conjugated

(C) Real-time qPCR showing *Gata6* induction does not occur until Nanog levels have been inhibited in response to FCXC<sup>T2</sup> induction.

(D) Real-time qPCR analysis of *Nanog* repression following 6 hr of FCXC<sup>T2</sup> induction in the presence or absence of cycloheximide (20  $\mu$ M) showing similar extents of repression with or without protein synthesis.

(E) Western blot showing ectopic expression of NANOG by transient transfection in cells containing FCXC<sup>T2</sup> 48 hr posttransfection and 24 hr after FCXC<sup>T2</sup> induction. Cells were transfected with a plasmid expressing a *Nanog* (long isoform) cDNA driven by a CAGs promoter. The following day, the medium was changed and FCXC<sup>T2</sup> induced for 24 hr. Cells transfected with an identical plasmid-expressing mCherry serving as a negative control.

(F) Real-time qPCR showing a reduction in PrE gene expression in cultures exogenously expressing Nanog.

(G) Real-time qPCR showing the effect of FCXC<sup>T2</sup> induction on expression of the pluripotency network was the same, regardless of whether Nanog was expressed in these cells. Nanog could increase the expression of these factors in control cultures but had no effect on Erk-mediated suppression of pluripotent gene expression. Data are presented as the mean  $\pm$  SD of independent clones.

(H and I) Real-time qPCR analysis of ESC (*Pre-Nanog* and *Esrrb*) and PrE (*Gata6*) expression in sorted populations from Figure S4B. NANOG-mCherry was again unable to sustain expression of pluripotency/epiblast markers; however, *Gata6* was now even more acutely repressed. Data are presented as the mean  $\pm$  SD of technical replicates.

See also Figure S4.

secondary antibodies (Alexa Fluor, Molecular Probes), visualized using a Chemidoc MP (Bio-Rad) and quantified using ImageJ. See Table S4 for a list of antibodies and concentrations used.

### Immunofluorescence

Cells were washed and fixed in 4% formaldehyde (Fisher Scientific, PI-28906), blocked, and permeabilized in 5% donkey serum and 0.3% Triton. Antibodies were incubated overnight in 1% BSA and 0.3% Triton in PBS and subsequently visualized with the appropriate secondary (Alexa Fluor, Molecular Probes).

### Flow Cytometry

Cells were dissociated with Accutase and incubated with the appropriate antibody in 10% FCS:PBS for 30 min, washed extensively, and analyzed on an LSRFortessa (BD Biosciences). Dead cells were excluded based on DAPI inclusion.

### Microarray Analysis

Transcriptome analysis was carried out in-house with one-color 8 × 60K Mouse Gene Expression Microarrays (Agilent Technologies, G4852A) as per manufacturer's instructions. Briefly, 150 ng of total RNA was labeled using the Low Input Quick Amp Labeling Kit (Agilent Technologies, 5190-2305). Labeled samples were hybridized overnight and then washed and scanned using the high-sensitivity protocol (AgilentG3\_HiSen\_GX\_1color) on a SureScan microarray scanner (Agilent Technologies), and probe intensities were obtained by taking the gProcessedSignal from the output of Agilent feature extraction software using default settings. Probe intensities were analyzed using the NIA Array Analysis tool (<http://lgsun.grc.nia.nih.gov/ANOVA/>).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.11.032>.

### AUTHOR CONTRIBUTIONS

W.B.H. and J.M.B. conceived the study. W.B.H. performed the experiments. W.B.H. and J.M.B. interpreted the data and wrote the paper.

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